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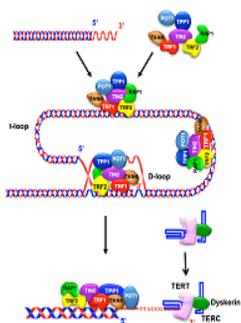
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STAT2 signaling and dengue virus infection

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Keywords: dengue virus, interferon, STAT2, NS5, antagonism, host tropism, mouse model

Abbreviations: DENV, dengue virus; IFN, interferon; DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; JEV, Japanese encephalitis virus; WNV, West Nile virus

Dengue virus (DENV) is an important human pathogen whose byzantine relationship with the immune response is poorly understood. DENV causes dengue fever and dengue hemorrhagic fever/dengue shock syndrome, diseases for which palliative care is the only treatment. DENV immunopathogenesis studies are complicated by the lack of an immunocompetent small-animal model, and this has hindered anti-DENV drug and vaccine development. This review describes strategies that DENV uses to evade the type I interferon response and focuses on how data gained from the study of DENV NS5-mediated STAT2 degradation may be used to create immunocompetent DENV mouse models and design anti-DENV therapeutics.

Introduction

Dengue virus (DENV) causes most of the world's mosquito-borne viral infections, and is the etiologic agent of dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). One of the first lines of host immunity against DENV is the type I interferon (IFN-I or IFN α/β) response, which inhibits viral replication, and sets the stage for the development of adaptive immunity. DENV-mediated degradation of signal transducer and activator of transcription 2 (STAT2), a component of the IFN α/β signaling pathway, has emerged as an important determinant of DENV pathogenesis and host tropism. Here we review the strategies that DENV uses to evade the type I interferon response, and postulate how studying DENV NS5-mediated STAT2 degradation may contribute to the development of immunocompetent DENV mouse models and anti-DENV therapeutics.

Dengue Disease and Dengue Virus

There are over 50 million DENV infections and approximately 500 000 cases of DHF/DSS annually.¹ More than 2.5 billion people live in the warm climates that are home to expanding populations of *Aedes aegypti* and *Aedes albopictus* mosquitoes, the vectors of DENV.¹ Many DENV infections are asymptomatic or show only mild symptoms but DF and DHF/DSS occur in a subset of patients. Older names for DF include “coup de barre” (“beating with a stick”) and “break bone fever” to describe the intense headache, myalgia and bone pain that accompany the disease.²⁻⁴ Other symptoms of DF include subthreshold vascular permeability and increased liver enzymes. DF progresses to DHF/DSS when patients develop capillary leakage, thrombocytopenia and liver damage.²⁻⁴ Because host immunity is an important contributor to DENV pathogenesis,^{5,6} focusing on the interaction between DENV and the host immune response is a promising approach to the development of drugs and vaccines against DENV.

The recent discovery of DENV-5 brings the total number of known DENV serotypes to five.⁷ These five serotypes belong to the flavivirus genus of the *Flaviviridae* family. The flavivirus genus includes important arthropod-borne viruses such as West Nile virus (WNV) and Japanese encephalitis virus (JEV). All flaviviruses contain a capped single-stranded RNA genome. In addition to its role as the viral genetic material, the genome functions as an mRNA whose translation yields a polyprotein that is cleaved by host proteases and the viral NS2B/3 protease to produce the structural and nonstructural proteins of the virus (Fig. 1). There are three structural proteins, capsid (C), premembrane/membrane (prM/M), and envelope, and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Fig. 1). The nonstructural proteins mediate replication of the viral RNA and antagonism of the host immune response, while the structural proteins encapsulate newly copied viral RNAs into DENV virions.

DENV Replication and the Type I IFN Response

DENV replicates in a variety of human cell types including endothelial cells, fibroblasts, dendritic cells (DCs), macrophages, and B cells.⁸⁻¹³ Infection of these cells leads to activation of the type I interferon (IFN-I or IFN α/β) response, an innate immune

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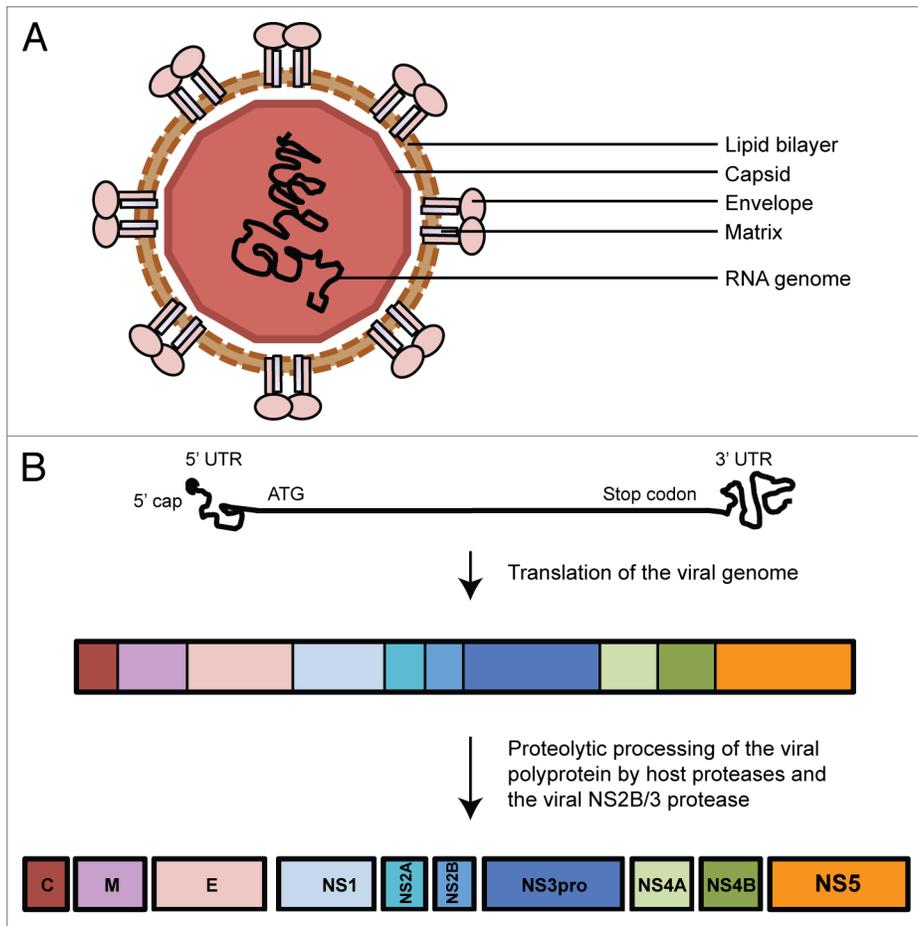


Figure 1. The DENV virion and genome. (A) DENV contains a capped plus-strand RNA genome that is surrounded by a shell composed of capsid (C) proteins. The capsid is enveloped by a lipid bilayer embedded with envelope (E) and membrane (M) proteins that mediate virus entry into susceptible cells. (B) The DENV genome functions as an mRNA whose translation yields a polyprotein that is processed by the viral NS2B/3 protease and host proteases to give the structural and nonstructural proteins of the virus.

mechanism that protects the host against viral infections. During viral replication, double-stranded RNA and other pathogen-associated molecular patterns (PAMPs) accumulate within the cell. The recognition of PAMPs by pattern recognition receptors such as retinoic-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA-5), and Toll-like receptor 3 (TLR3) leads to signaling events that culminate in IFN α/β production.¹⁴ In vivo and cell culture experiments have shown that TLR3, RIG-I, and MDA-5 contribute to IFN production in response to DENV infection but that RIG-I and MDA-5 may serve redundant roles.^{13,15,16} DENV-infected cells produce far less IFN α/β than cells infected with more potent inducers of IFN α/β such as Sendai virus due to the cleavage of STING,¹⁷⁻²⁰ an adaptor protein that is believed to function downstream of MDA-5 and RIG-I as well as cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS), a sensor of cytoplasmic dsDNA.²¹ Despite this immune evasion mechanism, IFN production is not completely halted by NS2B/3 protease, and IFN α/β is secreted

from infected cells as DENV infection proceeds.¹⁷ These quantities of IFN α/β are enough to inhibit DENV replication.^{9,12,13,22}

IFN α/β signaling ensues when IFN α/β from an infected cell binds to type I IFN receptors (IFNAR1/2) found on the surface of the infected cell or nearby cells. IFNAR1/2 engagement leads to the activation of Janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2), two tyrosine kinases that physically associate with IFNAR1/2. Tyk2 and JAK1 phosphorylate signal transducer and activator of transcription 1 (STAT1) and signal transducer and activator of transcription 2 (STAT2), which interact with interferon regulatory factor 9 (IRF9) to form IFN-stimulated gene factor 3 (ISGF3), a complex that recognizes IFN-stimulated response elements (ISREs). Binding of ISGF3 to the ISREs of IFN-stimulated genes (ISGs) leads to the transcription of ISGs (Fig. 2A).²³ Several ISGs encode proteins with anti-DENV activity. For example, interferon-induced transmembrane proteins 1, 2, and 3 (IFITM1, IFITM2, and IFITM3) inhibit early steps in dengue replication while viperin, interferon-stimulated gene 20 (ISG20), and dsRNA-activated kinase (PKR) inhibit the synthesis of DENV macromolecules.²⁴⁻²⁷ Previously uncharacterized ISGs such as IFN α -inducible protein 6 (IFI6), heparanase (HPSE), and N-ethylmaleimide-sensitive factor attachment protein α (NAPA) have also been identified as inhibitors of DENV replication.²⁸

Type I IFN Signaling Evasion by DENV

DENV and DENV replicons inhibit IFN α/β signaling in human cells.²⁹⁻³⁷ In fact, DENV has devoted a significant portion of its genome to antagonizing human type I IFN signaling. NS2A, NS4A, and NS4B have been shown to inhibit STAT1 phosphorylation while NS5 has been shown to mediate proteasome-dependent STAT2 degradation (Fig. 2A).^{31-35,37} However, these four viral proteins also have roles that are distinct from IFN α/β signaling antagonism. For example, NS2A is required for virion assembly, while NS4A and NS4B are required for induction of the membranes upon which viral replication occurs, and for organization of the replication complex, respectively.³⁸⁻⁴⁰ Expression of any of the three decreases ISRE promoter activation

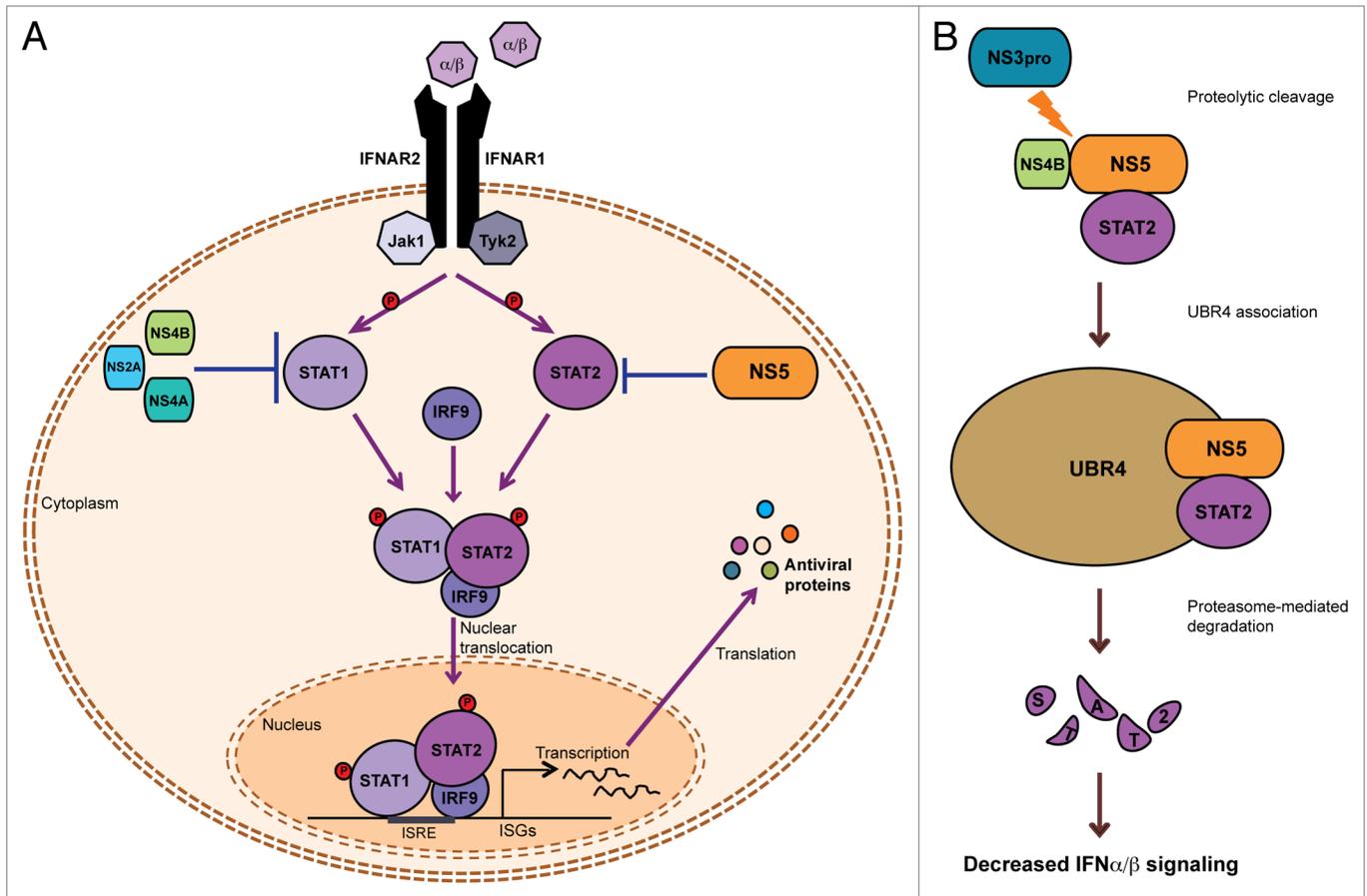


Figure 2. DENV antagonizes IFN α/β signaling. (A) IFN α/β engagement by IFNAR1/2 leads to the activation of Janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2), two tyrosine kinases that are associated with IFNAR1/2. Tyk2 and JAK1 phosphorylate signal transducer and activator of transcription 1 (STAT1) and signal transducer and activator of transcription 2 (STAT2), which interact with interferon regulatory factor 9 (IRF9) to form IFN-stimulated gene factor 3 (ISGF3), which recognizes IFN-stimulated response elements (ISREs). Binding of ISGF3 to ISREs leads to the transcription of IFN-stimulated genes (ISGs). Several ISGs, such as ISG20, viperin and IFITM1–3, encode proteins with anti-DENV activity. NS5 inhibits IFN α/β signaling by targeting STAT2 for degradation while the NS2A, NS4A and NS4B proteins inhibit STAT1 phosphorylation. (B) DENV-mediated STAT2 degradation requires proteolytic cleavage of NS5, which promotes its interaction with UBR4, a 600 kD host protein. The interaction of NS5, STAT2 and UBR4 are required for NS5-mediated, proteasome-dependent STAT2 degradation.

and STAT1 phosphorylation, but their combined expression has an even stronger inhibitory effect.³¹

NS5 is a multidomain protein that encodes many functions. The NS5 N-terminus encodes a methyl transferase that induces methylation of guanine N7 and 2-hydroxyl ribose of the viral RNA cap.^{41,42} These modifications are required for viral replication and for evading the antiviral IFN-induced tetratricopeptide repeat (IFIT) proteins, respectively.^{41,42} The N-terminus of NS5 also contains a guanylyltransferase, which is required for 5' RNA cap synthesis.⁴³ NS5's most studied role is that of the viral RNA-dependent RNA polymerase (RdRp), which is encoded by its C-terminal domain.^{44–46} NS5 mutants that are deficient in one or more roles while being proficient in others have been characterized,^{47,48} and it is thought that phosphorylation may serve as a switch among the various functions.⁴⁹ Over the past decade, flavivirus NS5 proteins have surfaced as potent antagonists of IFN signaling.^{34,35,50–54} However, NS5 proteins of different flaviviruses accomplish their IFN signaling inhibition in disparate ways. For example, WNV and JEV inhibit IFN signaling by preventing

phosphorylation of signaling proteins while DENV NS5 promotes the proteasomal degradation of human STAT2.^{34,37,51,54}

Degradation of STAT proteins is a common mechanism of virus-mediated IFN signaling inhibition. For instance, expression of the V proteins of human parainfluenza virus 2 (HPIV2) or parainfluenza virus 5 (PIV5) leads to proteasome-mediated degradation of STAT2 and STAT1, respectively.^{55,56} However, DENV NS5-mediated STAT2 degradation requires an extra step as compared with paramyxovirus-induced degradation. Unlike HPIV2 V, NS5 does not mediate STAT2 degradation when it is simply expressed exogenously from a plasmid.^{34,35} Instead NS5 has to be expressed as part of a larger precursor protein that is then proteolytically cleaved to yield a STAT2-degradation-competent NS5 (Fig. 2B).³⁴ During a DENV infection, NS2B/3 protease cleaves NS5 away from NS4B, and it is this processed NS5 that facilitates STAT2 degradation.³⁴ STAT2 degradation also proceeds when an NS5 construct containing a tobacco etch virus (TEV) protease cleavage site at its N-terminus is expressed in cells expressing TEV protease,

suggesting that it is cleavage alone and not the identity of the protease catalyzing the cleavage that determines if NS5 is able to efficiently mediate STAT2 degradation.³⁴ In fact, our group was able to take advantage of this unique feature to engineer an NS5 construct that would effectively mediate STAT2 degradation when expressed in cells. When DENV NS5 is engineered with an ubiquitin moiety fused to its N-terminus (ubiquitin-NS5), cellular hydrolases cut ubiquitin away from NS5 similarly to how the viral protease cuts NS4B away from NS5 during DENV infection.³⁴ When we purified NS5 from human cells expressing tagged ubiquitin-NS5, we identified a 600 kD host protein known as UBR4.³⁷ UBR4 binds preferentially to proteolytically-processed DENV NS5 over unprocessed DENV NS5 but does not bind to other flavivirus NS5 proteins (**Fig. 2B**). DENV-mediated STAT2 degradation and DENV replication decrease when UBR4 levels are reduced by RNA interference in interferon-competent primary dendritic cells and cell lines.³⁷ However, decreasing UBR4 levels in cells that cannot produce IFN α/β does not affect DENV replication unless exogenous IFN α/β is added to these cells.³⁷ Thus UBR4 is required by NS5 to antagonize IFN α/β signaling but does not appear to be necessary for other aspects of DENV replication. Though UBR4 does not contain a known E3 ligase motif like a HECT or RING domain, it is a member of the N-recogin/UBR family, which contains several confirmed E3 ligases.⁵⁷ Whether UBR4 functions as an E3 ligase or as the recognition subunit of a larger E3 ligase complex is currently under investigation, but its identification as an important player in DENV-mediated STAT2 degradation lays the foundation for designing therapeutics that target the NS5/UBR4 interaction.

The interaction between NS5 and UBR4 may also inform DENV vaccine design. An ideal live vaccine is attenuated but immunogenic. Live vaccines such as the truncated NS1 influenza virus mutants that are defective at evading the IFN response, have been shown to safely induce immunity in animal models.⁵⁸ We have found that NS5/UBR4 interaction and DENV-mediated STAT2 degradation require amino acids threonine 2 and glycine 3 of NS5.³⁷ Mutation of these residues or others that prevent interaction of NS5 and UBR4 but leave other functions of NS5 intact could result in viruses that would be attenuated in humans due to their increased sensitivity to the effects of IFN α/β . Such viruses may function as effective DENV vaccines.

NS5-Mediated STAT2 Degradation is a Determinant of DENV Host Tropism

DENV is unable to replicate in wild-type mice but it can replicate in mice that have defects in IFN signaling due to the absence of IFN α/β receptors or one or more STAT proteins.^{15,59-61} Our group has shown that a major reason that DENV replicates efficiently in human but not mouse cells is DENV's ability to subvert human but not murine IFN α/β signaling. DENV NS5 can bind human STAT2 but is unable to bind murine STAT2, and as a consequence, mouse STAT2 is not degraded in DENV-infected mouse cells.⁵⁹ In a side-by-side comparison of the effects

of human STAT2 and mouse STAT2 in STAT2-deficient human or murine cells, IFN α/β was able to inhibit DENV replication in cells expressing murine STAT2 but not in cells expressing human STAT2.⁵⁹ Furthermore, DENV NS5 binds murine UBR4 in murine cells.³⁷ This suggests that replacing murine STAT2 with human STAT2 in mice could potentially result in an immune-competent animal that would permit DENV replication, especially as murine and human STAT2 are interchangeable in the type I IFN signaling cascade.⁶² In the mouse model of PIV5, transgenic expression of human STAT2 permits parainfluenza virus 5 V protein to evade IFN signaling by binding STAT1.⁶³ This strategy would be unsuitable for DENV however, because murine STAT2 would still be available to transmit signals within the cascade.

Though STAT2 knockout mice support DENV replication, they do not develop hemorrhagic disease.^{15,34} Serious dengue illness occurs only in mice that lack components of both type I and type II IFN signaling, indicating that a good mouse DENV model may require modification of type II IFN signaling components or downstream effectors in addition to replacing murine STAT2 with human STAT2. Two recent papers suggest that DENV tropism is also determined at the level of IFN production.^{18,20} NS2B/3 protease cleaves human but not murine STING and this results in increased type I IFN production and lower DENV replication in mouse vs. human cells.^{18,20} Thus other pathways may also need to be modified to create the ideal DENV mouse model. A clonal immunocompetent mouse model would be an improvement on the currently available DENV models: AG129 mice and mice humanized with CD34⁺ human cells. AG129 mice lack type I and type II IFN receptors and are therefore not amenable to studying how the immune system participates in DENV disease. Though humanized mice develop a human-like system, engraftment of CD34⁺ human cells is variable and can lead to considerable variation from mouse to mouse.^{64,65} It is likely that a human STAT2 knock-in mouse would have a functional immune system but would also permit enough DENV replication to allow for the isolation of DENV variants that are better at evading later blocks to viral replication. This would expedite the identification of additional determinants of DENV pathogenesis thus providing new targets for rational drug design.

Conclusions

A large portion of the DENV genome is devoted to encoding proteins such as NS2B/3 protease, which inhibit IFN α/β expression, and NS5, NS4B, NS2A, and NS4A, which inhibit IFN α/β signaling. The NS5 protein of all flaviviruses tested so far have been shown to antagonize IFN signaling by completely different mechanisms suggesting that the NS5 IFN signaling antagonism function arose independently several times throughout evolution. It is likely that the NS5 protein acquired the IFN signaling inhibition function in each case because the polyprotein-based strategy of flaviviral protein expression results in excess expression of NS5 even though only small amounts are needed for polymerase and transferase functions. The study of DENV-mediated IFN

antagonism and NS5-mediated STAT2 degradation in particular has offered insights that could guide the development of an immunocompetent mouse model of dengue disease. These findings may also be useful for designing anti-DENV vaccines and drugs. Continued research into the immune evasion strategies of

DENV is expected to yield impactful tactics for fighting DENV in the coming years.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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